



# Comparative calorimetric and spectroscopic studies of the effects of cholesterol and epicholesterol on the thermotropic phase behaviour of dipalmitoylphosphatidylcholine bilayer membranes

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## ABSTRACT

We carried out comparative differential scanning calorimetric and Fourier transform infrared spectroscopic studies of the effects of cholesterol (Chol) and epicholesterol (EChol) on the thermotropic phase behaviour and organization of dipalmitoylphosphatidylcholine (DPPC) bilayers. EChol is an epimer of Chol in which the axially oriented hydroxyl group of C3 of Chol is replaced by an equatorially oriented hydroxyl group, resulting in a different orientation of the hydroxyl group relative to sterol fused ring system. Our calorimetric studies indicate that the incorporation of EChol is more effective than Chol in reducing the enthalpy of the pretransition of DPPC. EChol is also initially more effective than Chol in reducing the enthalpies of both the sharp and broad components of the main phase transition of DPPC. However, at higher EChol concentrations (~30–50 mol%), EChol becomes less effective than Chol in reducing the enthalpy and cooperativity of the main phase transition, such that at sterol concentrations of 50 mol%, EChol does not completely abolish the cooperative hydrocarbon chain-melting phase transition of DPPC, while Chol does. However, EChol does not appear to form a calorimetrically detectable crystallite phase at higher sterol concentrations, suggesting that EChol, unlike Chol, may form dimers or lower order aggregates at higher sterol concentrations. Our spectroscopic studies demonstrate that EChol incorporation produces more ordered gel and comparably ordered liquid-crystalline bilayers compared to Chol, which are characterized by increased hydrogen bonding in the glycerol backbone region of the DPPC bilayer. These and other results indicate that monomeric EChol is less miscible in DPPC bilayers than is Chol at higher sterol concentrations, but perturbs their organization to a greater extent at lower sterol concentrations, probably due primarily to the larger effective cross-sectional area of the EChol molecule. Nevertheless, EChol does appear to produce a lamellar liquid-ordered phase in DPPC bilayers.

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## 1. Introduction

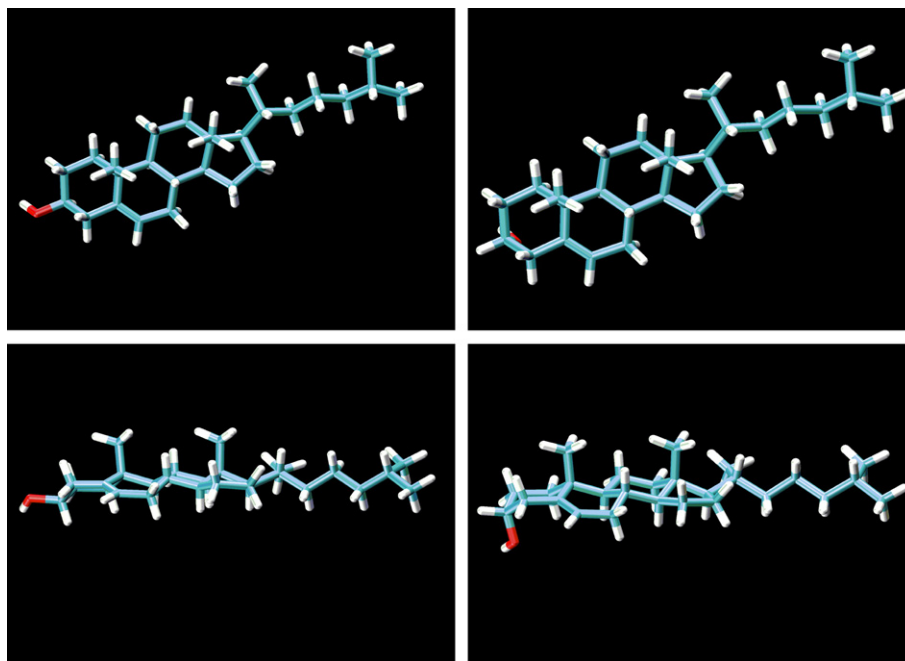
Cholesterol (Chol) is a major and essential lipid component of the plasma membranes of the cells of higher animals and is also found in lower concentrations in certain intracellular membranes in vesicular communication with the plasma membrane (see refs.[1–3]). Although

Chol has a number of different functions in animal cells, its primary role is as a modulator of the physical properties and lateral organization of the plasma membrane lipid bilayer. Thus, many studies of the interaction of Chol with phospholipid monolayer and bilayer model membranes have been performed, utilizing a wide range of physical techniques (see refs.[2,4–7]). These studies, most of which have utilized symmetrical chain, linear saturated PCs, have established that one of the major effects of Chol incorporation on phospholipid monolayer and bilayer model membranes is a broadening and eventual elimination of the cooperative gel-to-liquid-crystalline ( $L_{\beta}/L_{\alpha}$ ) phase transition and its replacement by a phase with an intermediate degree of organization. Thus, in the  $L_{\alpha}$  phase, which would exist at physiological temperatures in biological membranes in the absence of sterols, the presence of Chol significantly increases the orientational order of the phospholipid hydrocarbon chains and decreases the cross-sectional area occupied by the phospholipid molecules, while restricting the rates of phospholipid lateral diffusion or hydrocarbon chain motion only moderately. As well, the presence of Chol increases both the thickness and mechanical strength

**Abbreviations:** Chol, cholesterol; EChol, epicholesterol; Lano, lanosterol; PC, phosphatidylcholine; SpM, sphingomyelin; PE, phosphatidylethanolamine; DPPC, dipalmitoylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; OPPC, 1-oleoyl-2-palmitoylphosphatidylcholine; MLVs, multilamellar vesicles; LUVs, large unilamellar vesicles; SUV, small unilamellar vesicles; DSC, differential scanning calorimetry; NMR, nuclear magnetic resonance; ESR, electron spin resonance; FTIR, Fourier transform infrared;  $T_p$ , pretransition temperature;  $T_m$ , main transition temperature;  $\Delta H$ , transition enthalpy;  $\Delta T_{1/2}$ , width of the phase transition at half height, inversely related to the cooperativity of the phase transition;  $L_{\beta}'$  and  $L_{\alpha}'$ , lamellar gel phases with tilted and untilted hydrocarbon chains, respectively;  $P_{\beta}'$ , rippled gel phase with tilted hydrocarbon chains;  $L_{\alpha}$  or  $L_d$ , lamellar liquid-crystalline or liquid-disordered phase;  $L_o$ , lamellar liquid-ordered phase;  $H_{II}$ , inverted hexagonal phase

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**Fig. 1.** Molecular models of Chol and EChol. The top panels show views normal to the plane of sterol ring and the bottom panels show views parallel to the plane of the sterol ring. The two sterols only differ in the orientation of their hydroxyl groups (red) at C3. The molecules were minimized using DSviewer 6.0 (Accelrys Software Inc., San Diego, CA).

and decreases the permeability of the phospholipid bilayer in the physiologically relevant  $L_{\alpha}$  phase. The relatively high rates of intramolecular and intermolecular motion characteristic of phospholipid model membranes in the presence of higher levels of Chol, coupled with an increased hydrocarbon chain order and a decreased area compressibility, have prompted several workers to postulate the existence of a thermodynamically discrete liquid-ordered ( $L_o$ ) phase in model and biological membranes [5,8,9]. However, as many of the physical properties of model membranes composed of Chol and a single phospholipid change smoothly and monotonically with progressive increases in Chol concentration up to 50 mol% (see refs. [2,4–7]), the existence of thermodynamically discrete, macroscopic  $L_o$  and  $L_{\alpha}$  phases in such binary systems has been questioned (see refs. [7,10–11]), and it has been suggested that the behaviour of phospholipid/Chol systems can be explained by the formation of various superlattices [12] or molecular complexes [13]. However, in model membranes composed of Chol, unsaturated PCs and natural SpMs, the specificity of the interaction of Chol for SpM can result in the formation of macroscopic domains enriched in Chol and SpMs and depleted in unsaturated PCs, and such domains are thought to form the molecular basis for the possible existence of detergent-insoluble, Chol- and SpM-enriched lipid rafts in biological membranes (see refs. [14–19]). Even in such ternary systems, however, the existence of thermodynamically discrete  $L_o$  and  $L_d$  systems has not been detected by DSC or X-ray diffraction [20], and a number of workers have found the evidence for the existence of relatively large, long-lived lipid rafts in biological membranes unconvincing (see refs. [21–23]). Whatever the ultimate resolution of these outstanding questions, it is clear that the presence of Chol in biological membranes does modulate a number of different membrane functions, either directly or via its effects on the properties and lateral organization of the phospholipid bilayer (see refs. [2,24–26]).

A number of researchers have investigated the effects of systematic variations in the structure and stereochemistry of the Chol molecule on the thermotropic phase behaviour, organization, and passive permeability of phospholipid bilayers (see refs. [2,4,6,7]). In general, most structural and stereochemical alterations result in some loss of

the ability of the Chol molecule to produce its characteristic effects on phospholipid bilayers. In general, sterols must possess an equatorially oriented C3-hydroxyl group, a rigid planar fused ring system, and a flexible hydrocarbon side chain at C17 for maximum effect, whereas the degree of unsaturation of the ring system and the size of the alkyl side chain are of less importance. Interestingly, exactly the same structural features are required for exogenous sterols to support the maximum growth of sterol-auxotrophic mycoplasma, yeast, and mammalian cells (see refs. [24–26]), confirming that one of the major roles of Chol in eukaryotic membranes is to regulate the physical properties of the lipid bilayer.

EChol is perhaps the closest structural analog of Chol and, as such, has been extensively utilized in a variety of biophysical and biochemical studies of the role of sterols in model and biological membranes (see below). EChol is an epimer of Chol in which the axially oriented hydroxyl group at C3 is replaced by an equatorially oriented hydroxyl group, resulting in a different orientation of the hydroxyl group relative to the long axis of the sterol molecule (see Fig. 1). In Chol, molecular modeling shows that the C3- $\beta$ -hydroxyl group is aligned parallel to the long axis of the planar steroid nucleus, whereas in EChol the C3- $\alpha$ -hydroxyl group is aligned perpendicular to the steroid ring. Thus, when Chol is incorporated into phospholipid bilayers, the hydroxyl group projects away from the steroid nucleus and is located closer to the polar/apolar interfacial region of the membrane. In contrast, when EChol is incorporated into phospholipid bilayers, the hydroxyl group would project toward the  $\alpha$ - or smooth face of the steroid nucleus and would probably be located more deeply within the phospholipid bilayer, unless the entire EChol molecule were to be vertically displaced toward the interfacial region. Also, it would appear that the  $\alpha$ -hydroxyl group of EChol would not be able to undergo as extensive H-bonding interactions with the carbonyl ester and phosphate ester groups of adjacent phospholipid molecules or with interfacial water molecules as would Chol, unless the EChol molecule were tilted relative to the phospholipid bilayer membrane plane to a greater degree than Chol. Indeed, as we discuss below, some experimental support for an altered location and tilting of EChol relative to Chol in phospholipid bilayers is available in the literature.

The results of comparative studies of the interactions of Chol and EChol with model phospholipid bilayer and with various biological membranes vary considerably and often are not in agreement (see [4]). For example, although Demel et al. [27] state that the cross-sectional areas of Chol and EChol are roughly comparable in pure monolayer films deposited on water, their actual data indicates that EChol occupies a considerably larger area/molecule, particularly at higher temperatures, and a considerably reduced collapse pressure. In contrast, Stottrup and Keller [28] report a smaller area/molecule for Chol and a larger value for EChol at comparable lateral pressures and a much smaller difference in collapse pressures, whereas Stine et al. [29] report essentially identical cross-sectional areas for both sterols but a modestly reduced collapse pressure for EChol. Similarly, Demel et al. [27] report that EChol is much less effective than Chol in condensing monolayers composed of equimolar mixtures of DPPC/sterol, particularly at 37 °C, whereas Stottrup and Keller [28] find that EChol is actually more effective at reducing the average cross-sectional area of DPPC/sterol monolayers up to 30 mol% sterol, but is less effective at sterol concentrations of 40 mol% or more. Finally, Stine et al. [29] report that EChol is only moderately less effective than Chol in condensing DMPC/sterol monolayers at room temperature.

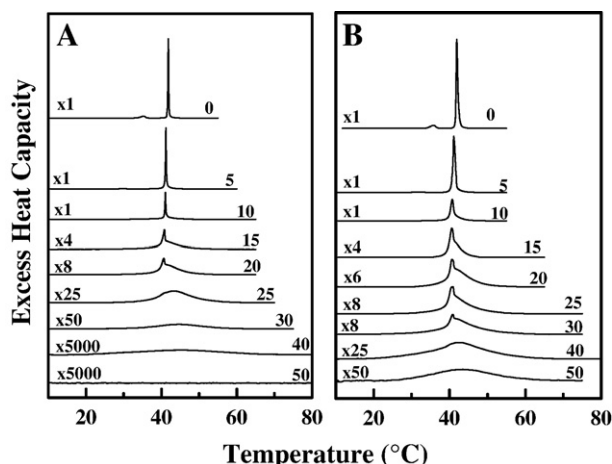
A number of comparative magnetic resonance spectroscopic studies of binary mixtures of Chol or EChol with phospholipid bilayers have been published. Dufourc et al. [30], using  $^2\text{H}$ -NMR to study both the behaviour of deuterated sterol molecules and the deuterated *sn*-2-myristoyl chain of DMPC/sterol mixtures, reported that Chol is only slightly more effective than EChol at ordering the fatty acyl chains of DMPC at higher temperatures and moderately less effective at disordering such chains at lower temperatures. However, the differential effects of Chol and EChol on the ordering of the DMPC hydrocarbon chains increase at higher temperatures. Moreover, Chol itself exhibits a higher order parameter than EChol at physiological temperatures, indicating that the time-averaged orientation of the Chol molecules is perpendicular to the bilayer plane, while the EChol molecule is tilted, although the latter assumes a less tilted orientation at higher temperatures. In contrast, a  $^2\text{H}$ -NMR study by Murari et al. [31] of deuterated sterols in DPPC bilayers found that Chol is tilted at an angle of 16–18° at both low and high temperatures, while EChol is aligned parallel to the bilayer normal at lower temperatures, but is tilted by 16–18° at higher temperatures. Lastly, a  $^{13}\text{C}$ -NMR study by Brainard and Cordes [32], reported that Chol and EChol exhibit essentially identical dynamics at physiological temperatures and have essentially identical effects on the dynamics of the host phospholipid molecules; however, at higher temperatures, EChol exhibits less restricted motion than Chol and also restricts the motion of adjacent egg PC molecules to a lesser extent. In contrast, an ESR study by Butler et al. [33] of large MLVs, composed of the phospholipids from the white matter of bovine brain or from human erythrocyte ghosts and various amounts Chol or EChol and a cholestane molecule spin labelled at C3, found that Chol strongly orders the sterol spin label but that EChol is without effect. Moreover, a later ESR study of egg PC/sterol MLVs by Hsia et al. [34], utilizing both cholestane and 12-doxyl stearic acid probes, reported the same results. Thus, these NMR and ESR studies differ greatly, particularly on the question of whether EChol is almost as effective as Chol in ordering the hydrocarbon chains of phospholipid bilayers or is completely ineffective in this regard.

A number of comparative studies of the effects of Chol and EChol incorporation on the passive permeability of model lipid bilayer and one biological membrane have also appeared, with somewhat mixed results. Demel et al. [35] originally reported that increasing the concentration of Chol drastically reduces the rate and extent of leakage of glucose from sonicated egg PC SUVs, essentially abolishing such leakage at 50 mol% sterol, while the incorporation of increasing amounts of EChol decreases glucose leakage only slightly, even at high levels of sterol incorporation. Similarly, the rate of glycerol entry into egg PC SUVs is reduced to a much greater extent by the incorporation

of 50 mol% Chol than by the same amount of EChol. However, EChol is almost as effective as Chol at reducing  $\text{Rb}^+$  release from these same vesicles, despite the fact that the maximum extent of EChol incorporation achieved in such vesicles was less than a third that of Chol. Bittman and Blau [36] later found that Chol and EChol have rather similar effects on reducing the water permeability of sonicated SUVs prepared from egg PC/sterol mixtures, although EChol is somewhat less effective than Chol at all sterol concentrations studied. Later still, Clejan et al. [37] reported that glucose release from SUVs prepared from a dialkyl PC and 30 mol% sterol is reduced by about half when Chol is present, but that the rate of glucose release is actually increased slightly when EChol is present. Finally, de Kruffy et al. [38] found that the incorporation of Chol into the membranes of *A. laidlawii* reduces the rate of glycerol and erythritol permeation by about half, but that the incorporation of similar amounts of EChol is without effect.

A number of other types of studies of the differential behaviour of Chol and EChol are also available. For example, both Nakagawa et al. [39] and Kan et al. [40] report the EChol exhibits more than a 10-fold higher rate of spontaneous exchange between sonicated egg PE and DPPC vesicles, respectively, than does Chol, indicating a much weaker interaction with the host phospholipid bilayer. On the other hand, Xu and London [41] found that EChol is almost as effective as Chol in inducing the formation of  $\text{L}_\alpha$  domains in large MLVs composed of ternary mixtures of DOPC/DPPC/sterol, and similar results were later presented by Beattie et al. [42] for giant unilamellar vesicles containing DOPC/DPPC/sterol. Similarly, Stottrup and Keller [28] found that the ability of EChol to induce immiscible liquid phases in DPPC monolayers is only slightly lower than that of Chol. However, Cheetham et al. [43] report that EChol is more effective than Chol at depressing the  $\text{L}_\alpha/\text{H}_{\text{II}}$  phase transition of large MLVs composed of unsaturated PE/sterol mixtures, indicating that EChol is more effective at destabilizing the lamellar liquid-crystalline phase relative to the inverted nonlamellar phase of this phospholipid. Finally, a molecular dynamics study of a DMPC/sterol bilayer by Rog and Pasenkiewicz-Gierula [44] indicates that EChol and Chol both increase the hydration of the bilayer surface and order and condense the bilayer, although these effects overall are somewhat weaker for EChol than for Chol. Interestingly, the tilt of EChol and Chol relative to the bilayer plane was found to be essentially the same in this study. However, EChol was found to project further into the bilayer interface than Chol, such that the hydroxyl group of EChol is located, on average, in the region of the DMPC phosphate groups, while the hydroxyl group of Chol is located in the region of the ester carbonyl groups. However, one concern with this molecular simulation is that identical cross-sectional areas were assumed for both sterols, although model building and monolayer studies ([27,28], but see [29]) suggest a somewhat larger area for EChol than for Chol.

High-sensitivity DSC is a nonperturbing thermodynamic technique that has been proven of great value in studies of the effect of Chol and Chol analogs on the thermotropic phase behaviour of phospholipid and sphingolipid bilayers [11,45–52]. However, there appear to have been no high-sensitivity DSC studies of pure EChol/phospholipid model membranes to date. Moreover, the only low-sensitivity DSC study published [38] utilized the nonphysiological phospholipid OPPC, which has the usual saturated–unsaturated fatty acid positional specificity reversed and thus exhibits an accentuated fatty acid chain length asymmetry. As well, results from only a single sterol concentration (20 mol%) are reported, but the authors state that 32 mol% Chol abolishes the main phospholipid phase transition. However, these calorimetric results are quite different from those obtained in high-sensitivity DSC studies of disaturated or mixed-chain saturated–unsaturated PC/Chol mixtures, where the main phase transition is not abolished until Chol concentrations near 50 mol% are reached. Moreover, as we have shown previously [11,46], the use of a constant sample size and DSC instrumental sensitivity setting can result in a failure to detect and accurately monitor the less energetic and poorly

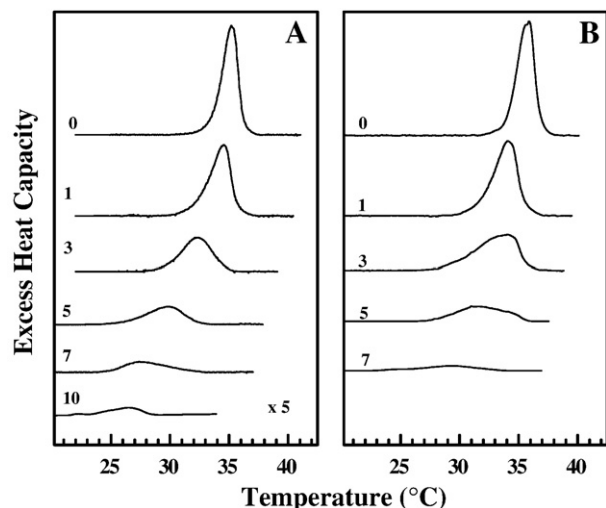


**Fig. 2.** DSC thermograms illustrating the effect of Chol (A) and EChol (B) on the gel/liquid-crystalline phase transition of DPPC. The thermograms shown were acquired at the sterol concentrations (mol%) indicated and have all been normalized against the mass of DPPC used. Y-axis scaling factors are indicated on the left hand side of each thermogram.

cooperative chain-melting phase transition occurring at high sterol concentrations. We have thus investigated the effect of Chol and EChol on the thermotropic phase behaviour of the well studied DPPC bilayers using a high-sensitivity calorimeter and an experimental protocol which ensures that the broad, lower enthalpy phase transitions occurring at higher sterol levels are accurately monitored. Moreover, we have also investigated the effects of Chol and EChol incorporation on the organization of DPPC bilayers by FTIR spectroscopy. Overall, our results indicate that the effects of EChol on the thermotropic phase behaviour and organization of DPPC vesicles are qualitatively similar but quantitatively different from those of Chol.

## 2. Materials and methods

The DPPC and Chol (>99% pure) were both obtained from Avanti Polar Lipids Inc. (Alabaster, AL), whereas the EChol (99% pure) was supplied by Steraloids Inc. (Newport, RI). Those materials were used without further purification. All organic solvents were of at least analytical grade quality and were redistilled before use. The preparation of samples for DSC was identical to that reported earlier [52]. Briefly, the samples for DSC experiments were prepared by mixing appropriate volumes of stock solutions of DPPC dissolved in chloroform with those containing the sterol. After drying under nitrogen, the dried phospholipids/sterol film was hydrated with 1 ml of deionized water and was



**Fig. 3.** DSC thermograms illustrating the effect of Chol (A) and EChol (B) on the pretransition of DPPC. The thermograms shown were acquired at the sterol concentrations (mol%) indicated and have all been normalized against the mass of DPPC used. The thermograms shown for DPPC/sterol mixtures containing 10 mol% Chol and 6.25 mol% EChol are plotted on 5-fold expanded y-axes relative to all others.

vigorously vortexed at 50–60 °C. The dispersion was then degassed and 324  $\mu$ L aliquots were withdrawn for DSC analyses. To ensure better resolution of the broad, low-enthalpy thermotropic transitions exhibited by sterol-rich mixtures, the amount of phospholipid used for DSC measurements was progressively increased with the sterol content of the mixture (see [46]). Typically, samples containing 1–3 mg phospholipid were used at sterol concentrations below 5 mol%, 5–8 mg phospholipid at sterol concentrations between 5 and 15 mol%, and 10–15 mg of phospholipid at all higher sterol concentrations. The reference cell always contained 324  $\mu$ L deionized, degassed water. DSC heating and cooling thermograms were recorded with a high-sensitivity Nano DSC (Calorimetry Sciences Corporation, Lindon, UT) operating at a scan rate of 10 °C/h. The data acquired were analyzed and plotted with the Origin software package (OriginLab Corporation, Northampton, MA). In cases where the DSC thermograms appeared to be a summation of overlapping components, the midpoint temperatures, areas and half-widths of the components were estimated with the aid of the Origin non-linear least squares curve- and peak-fitting procedures and a custom-coded function based on the assumption that the observed thermogram was a linear combination of components, each of which could be approximated by a reversible two-state transition at thermodynamic equilibrium. At least two independently prepared samples were analyzed for each sterol and each sterol concentration investigated and at least three DSC heating scans were performed on each sample.

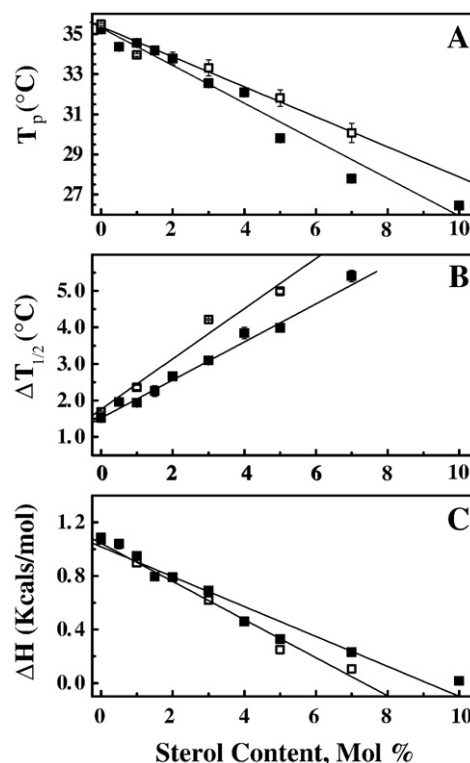
Samples used for FTIR spectroscopic experiments were prepared by dispersing dried lipid/sterol mixtures containing 2–3 mg of phospholipid in 50  $\mu$ L of distilled water at temperatures near 55–60 °C. The paste so formed was then sealed as a thin (25- $\mu$ m) film between the CaF<sub>2</sub> windows of a heatable, demountable liquid cell equipped with a 25- $\mu$ m Teflon spacer. Once mounted in the sample holder of the instrument, the sample temperature could be controlled between 20° and 90 °C by means of an external, computer-controlled water bath. FTIR spectra were acquired with a Digilab FTS-40 Fourier transform spectrometer (Biorad, Digilab Division, Cambridge, MA) using data-acquisition and data-processing protocols described by Lewis et al. [53].

## 3. Results

### 3.1. Differential scanning calorimetry studies

#### 3.1.1. The overall pattern of thermotropic phase behaviour observed in Chol/DPPC and EChol/DPPC dispersions

DSC heating scans of DPPC dispersions containing differing concentrations of either Chol or EChol are shown for comparative purposes in Fig. 2. The overall pattern of behaviour seen on heating in the



**Fig. 4.** The effect of increases in sterol concentration on the  $T_p$ ,  $\Delta H$  and  $\Delta T_{1/2}$  of the pretransition of DPPC. The symbols (—■—■—) and (—□—□—) represent the data from the Chol- and EChol-containing samples, respectively.



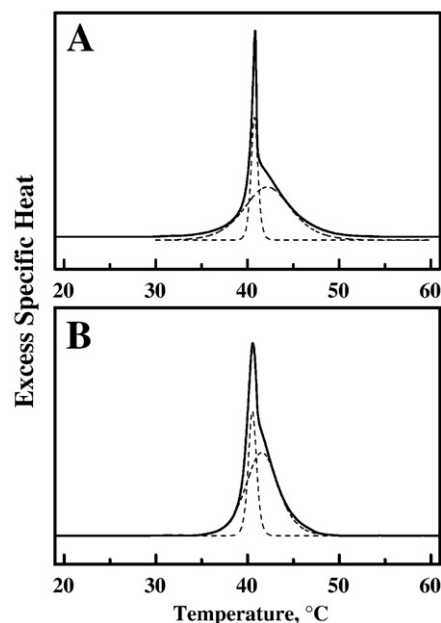
Chol/DPPC mixtures is similar to that reported previously using less sensitive DSC instruments [11], and the effects of EChol on the thermotropic behaviour of DPPC MLVs are broadly similar to that of Chol. In the absence of sterols, DPPC heating scans show two sharp endothermic peaks, initially centered at  $\sim 35^\circ\text{C}$  and  $\sim 41.9^\circ\text{C}$ , which correspond to the pretransition ( $L_\beta'/P_\beta'$ ) and main or chain-melting ( $P_\beta'/L_\alpha$ ) phase transitions, respectively. Increasing the sterol concentration gradually broadens the pretransition and reduces its temperature and enthalpy while decreasing its cooperativity in both sterol/DPPC mixtures. Similarly, for the main phase transition, increasing the sterol concentration initially produces a multicomponent endotherm, consisting of a sharp component that is progressively reduced in temperature, enthalpy and cooperativity, and a broad component that increases in both temperature and enthalpy but decreases in cooperativity. Thus, with increasing sterol concentrations, the sharp component disappears as the broad component grows. However, there are important quantitative differences in the effect of each sterol on the DPPC pretransition and on the two components of the main phase transition, as we discuss below.

### 3.1.2. The effects of Chol and EChol on the pretransition of DPPC

In order to investigate the disappearance of the DPPC pretransition in greater detail, we prepared sterol/DPPC mixtures covering a narrower range of either Chol or EChol concentrations. The gradual elimination of the pretransition in the DSC heating scans as a function of concentration for both Chol- and EChol/DPPC mixtures are shown in Fig. 3 and the derived thermodynamic measurements for both sterol/DPPC systems are presented in Fig. 4. In EChol/DPPC samples, both the magnitude of the reduction in  $T_p$  is less and its resultant rate of decrease with increasing sterol concentration (Fig. 4A) is less than the values obtained for the corresponding Chol-containing samples. Moreover, the pretransition endotherms of samples containing EChol are broader (Fig. 4B) and increase in width and also decrease in  $\Delta H$  (Fig. 4C) more rapidly than is observed in samples containing Chol. Thus, the pretransition is abolished at  $\sim 7$  mol% in EChol/DPPC mixtures, whereas it persists up to  $\sim 10$  mol% in corresponding samples containing Chol. Since these changes occur over a narrower range of sterol concentration in the EChol/DPPC samples (0–7 mol%) compared to the Chol/DPPC samples (0–10 mol%), we can conclude that EChol is more effective at abolishing the pretransition than is Chol on a molar basis. A possible molecular explanation for this observation will be presented in the Discussion.

### 3.1.3. The effects of sterol concentration on the main phase transition of DPPC

The DSC data shown in Fig. 2 indicates that at low to moderate sterol concentrations, both Chol- and EChol-containing DPPC bilayers exhibit asymmetric thermograms which consist of two overlapping thermal events. One of these components is considerably sharper than the other, its peak temperature and cooperativity decrease slightly, but its enthalpy decreases markedly with increasing sterol content. The other component is considerably broader, its midpoint temperature exhibits a more complex dependence on sterol content, and it is the only component persisting at the higher range of sterol concentrations. This pattern of sterol concentration-dependent behaviour has been observed previously in Chol/DPPC mixtures [11] and the resolved sharp and broad components have been ascribed to the differential melting of sterol-poor and sterol-rich lipid domains, respectively. However, there are significant similarities and differences between the sterol concentration-dependent behaviours exhibited by the Chol- and EChol-containing preparations, especially with regard to the quantitative aspects of the sterol concentration dependence of the overall properties of the underlying sharp and broad peaks (see Fig. 5). This aspect of our experimental observations was further examined through the application of computer-assisting curve- and peak-fitting procedures to deconvolve the observed DSC



**Fig. 5.** Illustration of the results typically obtained in our peak-fitting deconvolution analyses of the DSC thermograms exhibited by Chol-containing (A) and EChol-containing (B) DPPC bilayers. The thermograms shown both contained 15 mol% sterol. To facilitate visibility, the fitted curves are slightly displaced along the y-axis.

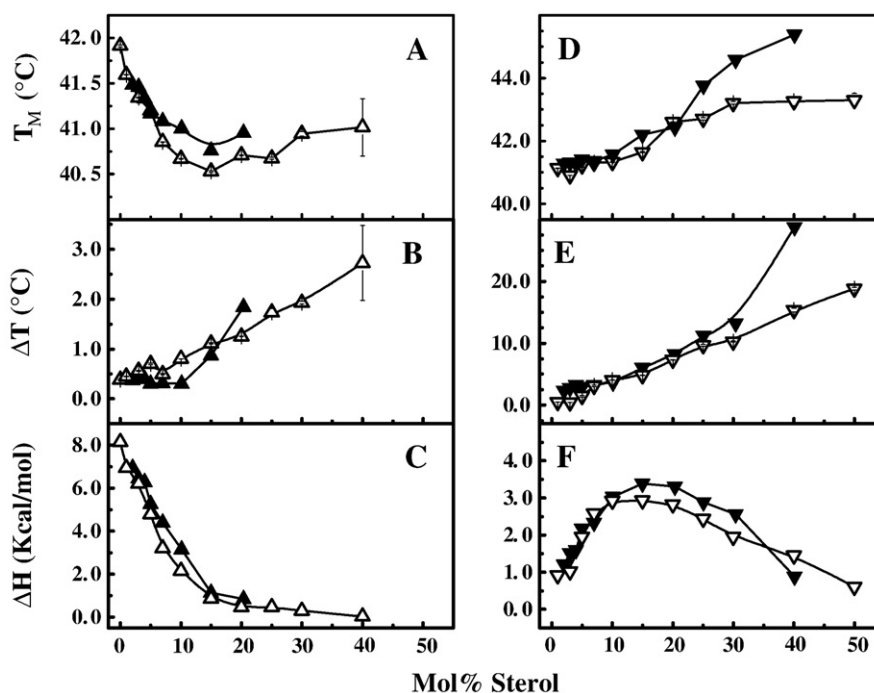
thermograms into their component peaks, so that the sterol-concentration dependence of each component could be examined and compared (see below).

The data presented in Fig. 5 is an example of the results typically obtained in our curve-fitting analyses of the DSC thermograms obtained for the gel/liquid-crystalline phase transition of Chol- and EChol/DPPC mixtures. These results indicate that over a lower sterol concentration range, the DSC thermograms obtained for the sterol/DPPC mixtures can be accurately simulated by a summation of two components (one sharp and one broad, see Fig. 5). Those observations, supported by the results of our FTIR spectroscopic studies, strongly suggest that DPPC bilayers containing lower Chol or EChol concentrations contain at least two significant populations of independently melting, sterol-poor and sterol-rich domains. At higher sterol concentrations, we find that the thermograms exhibited by both sterol/DPPC bilayers studied here can be accurately simulated by a single broad, low-enthalpy component. Below, we present a detailed comparison of the effects of variations in EChol and Chol content on the sharp and broad components from those DSC thermograms.

### 3.1.4. Effect of Chol and EChol on the sharp component of the DPPC main phase transition

The  $T_m$  values obtained from the sharp components of the main phase transition of the both sterol/DPPC mixtures are shown in Fig. 6A, B and C. Each sterol/DPPC system (Fig. 6A) shows a gradual decrease in  $T_m^{\text{shp}}$  with increasing sterol concentration, which closely follows the  $T_m$  values obtained for the overall DSC curves at lower sterol concentrations. Above 5 mol% sterol, the decrease in the  $T_m^{\text{shp}}$  values of both sterol/DPPC mixtures levels off and reaches a minimum at about 20 mol% sterol. Above this sterol concentration, a sharp component is no longer present in the Chol/DPPC mixtures, but the sharp component persists to sterol concentrations above 30 mol% in the EChol/DPPC mixtures.

The corresponding values of  $\Delta T_{1/2}^{\text{shp}}$  for both sterol/DPPC mixtures (Fig. 6C) indicate the EChol is initially more effective at broadening the sharp component of the main phase transition than is Chol, although at sterol concentrations above 10 mol%, the  $\Delta T_{1/2}^{\text{shp}}$  value increases more



**Fig. 6.** Thermodynamic parameters for the deconvolved sharp (A–C) and broad (D–F) components obtained from the DSC heating thermograms of the Chol ( $\blacktriangle$ ,  $\blacktriangledown$ ) and the EChol ( $\triangle$ ,  $\triangledown$ )/DPPC samples as a function of sterol concentration. The error bars were typically equal to or smaller than the size of the symbols.

rapidly with increasing concentrations of Chol than of EChol. At higher sterol concentrations, the  $\Delta T_{1/2}^{\text{shp}}$  values increase gradually and linearly for the EChol system, whereas those of the Chol/DPPC mixtures increase more steeply, reflecting the greater ability of Chol relative to EChol to broaden the DPPC main phase transition at higher sterol concentrations.

In contrast, the pattern of  $\Delta H_m^{\text{shp}}$  values for thermograms obtained from both sterol/DPPC samples is more complex (Fig. 6E). After the initial drop in enthalpy, there is a small window where the  $\Delta H_m^{\text{shp}}$  values for both sterol/DPPC mixtures are indistinguishable from one another and decrease very slowly (Fig. 6E). At sterol concentrations above 3 mol%, there is a more rapid decrease in the  $\Delta H_m^{\text{shp}}$  values for both sterol/DPPC mixtures. In the case of the EChol/DPPC system, the sharp component is abolished between 30 and 40 mol% sterol, whereas in the Chol/DPPC system it is abolished at ~25 mol%, again reflecting a greater ability of Chol to abolish the main phase transition of DPPC at higher sterol concentrations. The possible molecular basis for these differential effects will be addressed in the Discussion.

### 3.1.5. Effect of Chol and EChol on the broad component of the DPPC main phase transition

An analysis of the broad components obtained from our deconvolution of the overall DSC thermograms for both sterol/DPPC systems is shown in Fig. 6D, E and F. The broad component is initially found on the low temperature side of the sharp component in both sterol/DPPC systems and gradually increases in temperature with increasing sterol concentration. The broad component of the Chol/DPPC thermograms exhibits a monotonic increase in temperature over the entire range of sterol concentrations (Fig. 6D). Although the  $T_m^{\text{brd}}$  of the EChol/DPPC mixture also initially increases, above about 30 mol% sterol the  $T_m^{\text{brd}}$  of the EChol/DPPC samples reaches a maximum and does not significantly increase with additional increases in sterol concentration. The corresponding  $T_m^{\text{brd}}$  values for the Chol-containing samples continue to increase until 40 mol% sterol and at 50 mol% sterol the broad component of the DSC endotherm is abolished. The DSC thermograms clearly show that an endothermic peak is still present in samples containing 50 mol% EChol, but that it is abolished in corresponding samples containing the equivalent amount of Chol. Thus,

at lower sterol concentrations,  $T_m^{\text{brd}}$  behaves similarly in both sterol/DPPC systems, but differs above approximately 30 mol% sterol.

For both sterol/DPPC systems, the  $\Delta T_{1/2}^{\text{brd}}$  values are significantly larger than those of the sharp component and show a gradual increase with increasing sterol concentration. The  $\Delta T_m^{\text{brd}}$  values for both sterol/phospholipid systems are similar up to a sterol concentration of about 30 mol%. However, above that concentration, the  $\Delta T_{1/2}^{\text{brd}}$  values obtained from samples containing Chol increase significantly, whereas those of the corresponding EChol samples increase monotonically up to a concentration of 50 mol% sterol. This indicates that Chol has a greater broadening effect on the gel/liquid-crystalline phase transition of DPPC than does EChol but only at higher sterol concentrations.

The  $\Delta H_m^{\text{brd}}$  values of Chol/DPPC mixtures gradually increase up to a concentration of approximately 15 mol%, above which they steadily decrease until the endothermic peak is abolished at a sterol concentration of 50 mol%. The  $\Delta H_m^{\text{brd}}$  values of the EChol/DPPC samples follow a similar trend, reaching a maximum at about the same sterol concentration, but with slightly lower enthalpy values above a concentration of approximately 10 mol% EChol. In both sterol/DPPC systems, the enthalpy of the broad component crosses that of the sharp component at approximately 10 mol%, suggesting that at lower concentrations at least, both sterols interact with DPPC in a generally similar manner.

A comparison of the all three thermodynamic parameters for both the Chol and EChol/DPPC systems (Fig. 6) shows that the broad component is reduced more rapidly and thus is eliminated at a lower sterol concentration in the Chol/DPPC relative to the EChol/DPPC mixtures, suggesting either that Chol is better able to interact with the DPPC molecules present in the sterol-rich domain than is EChol, or that EChol is less soluble in DPPC bilayers at higher sterol concentrations, or both. This is supported by the plot of the overall enthalpy for both sterol/DPPC systems shown in Fig. 7, which indicates that EChol is more effective than Chol at reducing the enthalpy of the DPPC chain-melting phase transition at lower sterol concentrations, but is less effective in doing so at higher sterol concentrations.

A plot of the net temperature shift for both the sharp and broad components of both Chol- and EChol-containing samples is shown in

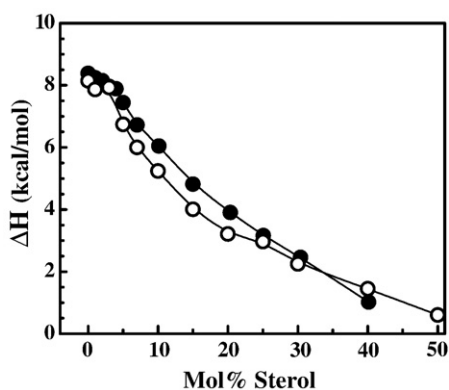


Fig. 7. Overall enthalpy values obtained for Chol (●) and EChol (○)/DPPC mixtures from DSC heating curves. The largest error bar was equal to the symbol diameter.

Fig. 8, the net temperature shift being defined as the temperature difference between the  $T_m$  and  $T_m^{shp}$  or  $T_m^{brd}$  components of the DSC thermograms. The temperature shift of the sharp component for the EChol/DPPC dispersions (Fig. 8A) is more negative than that of the corresponding Chol/DPPC values, but both show a similar dependence with increasing sterol concentration. The net temperature shift values for the broad component are similar up to a concentration of approximately 10 mol% sterol, but diverge with increasing sterol concentration. Chol clearly affects the  $T_m^{brd}$  more than does EChol at concentrations above 20 mol%. For EChol/DPPC mixtures, values reach a maximum above 25 mol%, whereas in the Chol samples, they continue to increase. The possible molecular basis for the observed differences in the thermotropic phase behaviour of the Chol/DPPC and EChol will be presented in the Discussion.

FTIR studies of DPPC alone, and DPPC/Chol and DPPC/EChol mixtures containing 30 mol% sterol, were carried out over a range of temperatures in order to permit a fuller understanding of the calorimetric data just presented. A sterol level of 30 mol% was chosen in order to maximize any effects of Chol and EChol on the organization of DPPC bilayer at temperatures above and below the main or chain-melting phase transition temperature region, while avoiding the potential problem with EChol immiscibility or aggregation in DPPC bilayers at higher sterol levels suggested by previous work and by our present DSC results. Using this nonperturbing spectroscopic technique, we investigated the effects of Chol and EChol incorporation on the gel/liquid-crystalline phase transition and the degree of DPPC

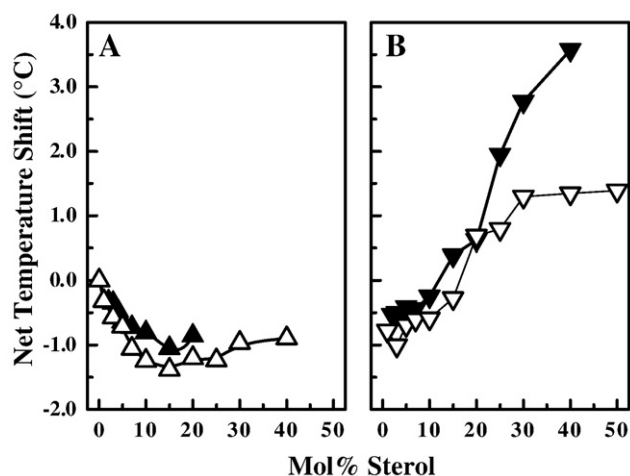


Fig. 8. The net temperature shift of the sharp and broad components of the main chain melting phase transition of Chol (▲,▼) and EChol/DPPC (△,▽) samples obtained from DSC heating thermograms relative to that of pure DPPC.

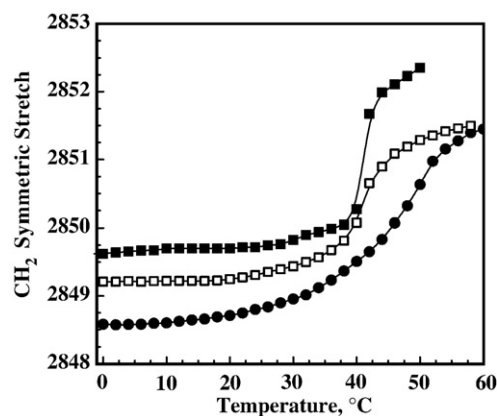
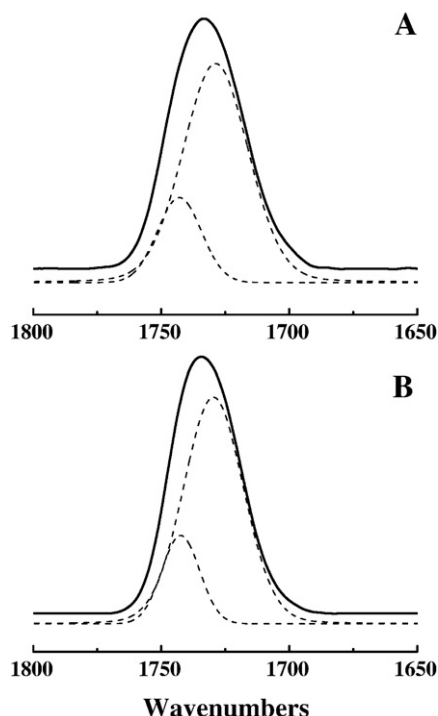


Fig. 9. Temperature-dependent changes of the  $CH_2$  symmetric stretching band maxima exhibited by sterol-free (■), cholesterol-containing (□) and epicholesterol-containing (●) DPPC bilayers. The sterol-containing bilayers contained 30 mol% sterol.

hydrocarbon chain rotational isomeric disorder by monitoring the frequency of the  $CH_2$  symmetric stretching band located near  $2850\text{ cm}^{-1}$ , the presence of solid-state DPPC hydrocarbon chain packing by the presence of the  $CH_2$  scissoring band near  $1468\text{ cm}^{-1}$ , the degree of hydrogen bond formation in the glycerol backbone region of the DPPC molecule by changes in the contours of the ester  $C=O$  stretching bands near  $1730\text{ cm}^{-1}$ , and changes in the polarity or hydrogen bonding state of the DPPC polar headgroup by monitoring the frequency of the  $O-P-O$  asymmetric stretching bands near  $1230\text{ cm}^{-1}$  (see [56–58]). We found that both 30 mol% Chol and EChol incorporation prevented the formation of the lamellar crystalline phase in DPPC bilayers incubated at low temperatures (data not presented), indicating that EChol, like Chol, is well dispersed in DPPC bilayers in the gel state [46]. Moreover, the incorporation of both sterols had no discernable effect on the  $O-P-O$  asymmetric stretching band, indicating that neither sterol had a significant effect on the polarity or hydrogen bonding state of the phosphate moiety of the DPPC polar headgroup. Therefore, only the effects of Chol and EChol incorporation on the organization of the DPPC hydrocarbon chains and glycerol backbone region will be considered in detail here.

The frequency of the  $CH_2$  symmetric stretching band maxima as a function of temperature for DPPC alone, and for DPPC/Chol and DPPC/EChol binary mixtures, are presented in Fig. 9. For DPPC itself, the  $CH_2$  symmetric band frequency is centered just below  $2850\text{ cm}^{-1}$  in the gel state, but increases sharply in frequency to about  $2852.5\text{ cm}^{-1}$  in the liquid-crystalline state. This increase in band width and frequency at the highly cooperative gel/liquid-crystalline phase transition of DPPC reflects increases in the mobility and gauche rotamer content of the DPPC hydrocarbon chains, respectively, accompanying the sharp chain-melting phase transition (see [54–56]). In contrast, in the DPPC/Chol and DPPC/EChol mixtures, the gel/liquid-crystalline phase transition is markedly broadened, particularly so for the latter system, as also observed by DSC (see Figs. 2 and 6). As well, the  $CH_2$  symmetric stretching frequency at low temperatures is lower in the EChol-containing than in the Chol-containing binary mixture, whereas at high temperatures their frequencies are comparable, and at both low and high temperatures the  $CH_2$  symmetric band frequencies fall below the values characteristic of pure DPPC bilayers. The simplest and most straightforward interpretation of these results is that both sterols order the DPPC hydrocarbon chains in the gel state, with EChol being more potent than Chol in this regard, while both sterols order the hydrocarbon chains of DPPC in the liquid-crystalline state in a comparable manner. However, we and others have pointed out that the  $CH_2$  symmetric stretching band frequency is not always a reliable indicator of hydrocarbon chain order in phospholipid/sterol and phospholipid/transmembrane peptide systems, particularly in the gel



**Fig. 10.** Representative FTIR spectra illustrating the components of the ester carbonyl stretching bands exhibited by DPPC bilayers at temperatures above (A) and below (B) the lipid gel/liquid-crystalline phase transition. The absorbance spectra shown were acquired at the temperatures indicated with the solid line representing the observed baseline corrected spectra and the dashed lines representing our estimates of the component bands as determined by a combination of Fourier-deconvolution and curve-fitting.

state (see [46,57] and references therein), so some caution may be in order in interpreting these experimental results too quantitatively, particularly at low temperatures. Nevertheless, since Chol and EChol are so similar in structure, these tentative interpretations of our experimental data seem generally reasonable for the liquid-crystalline state which exists at higher temperatures.

Illustrated in Fig. 10 are representative spectra which exemplify the temperature-dependent changes in the contours of sterol-free DPPC bilayers. It is now generally accepted that the ester carbonyl stretching bands in the infrared spectra of the gel and liquid-crystalline phases of lipids such as DPPC are resolvable into two components centered near 1728  $\text{cm}^{-1}$  and 1742  $\text{cm}^{-1}$  [58,59]. The lower-frequency component arises primarily from populations of H-bonded ester carbonyl groups and tends to be broader than the higher frequency component arising from populations of non H-bonded ester carbonyl groups [58,59]. Although changes in the overall center of gravity of the observed C=O stretching band are usually observed at the gel/liquid-crystalline phase transition of lipids such as DPPC, such observations are usually attributable to changes in the widths and relative intensities of the component bands, and not to significant changes in the frequencies of the component bands themselves [60]. We also find that the incorporation of ~30 mol% Chol and EChol into DPPC bilayers was accompanied by a downward shift (~2–4  $\text{cm}^{-1}$ ) in the centers of gravity of the phospholipid C=O stretching bands at all temperatures examined, and that observation is also the result of a relative decrease in the intensity of the C=O stretching band component located at 1742  $\text{cm}^{-1}$  and a relative increase in the intensity of component located at 1727  $\text{cm}^{-1}$ , along with changes in the intrinsic bandwidths of these bands. A more detailed examination of these spectroscopic changes is presented below.

Listed in Table 1 is a summary of the temperature-dependent changes in the component structure of the ester C=O stretching

bands exhibited by the sterol-free and sterol-containing DPPC bilayers examined. The data summarized therein reveal a number of interesting and structurally significant features. First, the melting of the hydrocarbon chains of the sterol-free and sterol-containing DPPC bilayers is always accompanied by an increase in the relative intensity of the lower-frequency component. Most probably, this reflects a phase-state-dependent increase in the relative size of the H-bonded population of ester carbonyl groups consistent with the expected increased penetration of water into polar/apolar interfacial regions of these bilayers at their respective hydrocarbon chain-melting phase transitions. However, we also find that incorporation of either Chol or EChol into DPPC bilayers is also accompanied by an increase in the relative intensity of the absorption bands arising from the H-bonded ester carbonyl groups, and that this effect is consistently slightly greater with the EChol-containing bilayers (see Table 1). This observation suggests that the presence of Chol and especially EChol increases the degree of H-bonding of the carbonyl groups located near the glycerol backbone region of the DPPC molecules. In principle, this sterol-induced increase in the degree of H-bonding could be due to additional H-bonding between the C3-hydroxyl of the Chol or especially the EChol molecule and the ester carbonyl groups of adjacent phospholipid molecules in the bilayer, or to an increase in the degree of water penetration into the glycerol backbone region of the DPPC bilayer, which may be facilitated by a sterol-induced increase in the spacing of the polar headgroups at the bilayer surface. However, these two possibilities cannot be distinguished with the FTIR spectroscopic data currently at hand.

Table 1 also shows that the line widths of the lower-frequency H-bonded population of ester carbonyl groups are consistently significantly greater than those of the higher frequency bands arising from the population of non-H-bonded ester carbonyl groups. Since both populations of ester carbonyl groups are summations of comparable contributions of *sn*1- and *sn*2-ester carbonyl groups [58,59], it seems unlikely that these differences in line width can be attributed to differences the mobilities of the free and H-bonded ester carbonyl groups themselves. Instead, the increased widths of the absorption bands of the H-bonded population are more likely to be reflecting heterogeneity in the nature, orientation and/or mobility of moieties H-bonded to ester carbonyl groups of the lipid. It is also apparent that the

**Table 1**

Characterization of the subcomponents of the ester C=O stretching bands exhibited by hydrated, sterol-free and sterol-containing DPPC bilayers

Peak maximum ( $\text{cm}^{-1}$ )	Peak width ( $\text{cm}^{-1}$ )	Peak area (% total)
<b>A. DPPC (<math>L_{\beta}</math> phase at 6 °C)</b>		
1728	27	76
1742	17	24
<b>B. DPPC (<math>L_{\alpha}</math> phase at 60 °C)</b>		
1728	30	82
1742	19	18
<b>C. DPPC+30 mol% cholesterol (liquid-ordered phase at 6 °C)</b>		
1728	30	80
1742	19	20
<b>D. DPPC+30 mol% cholesterol (liquid-disordered phase at 60 °C)</b>		
1728	33	84
1742	20	16
<b>E. DPPC+30 mol% epicholesterol (liquid-ordered phase at 6 °C)</b>		
1726	35	83
1742	19	17
<b>F. DPPC+30 mol% epicholesterol (liquid-disordered phase at 60 °C)</b>		
1726	36	89
1742	21	11

Parameter values are rounded to the nearest whole number.



gel/liquid-crystalline phase transition is itself accompanied by increases in the line widths of all components of the ester carbonyl band envelope. Most probably, this observation is largely reflecting thermally and/or phase-state induced increases in the mobility the carbonyl groups themselves, because it is also observed with all of the phospholipid absorption bands. Interestingly, we also find that the incorporation of either Chol or EChol into DPPC bilayers is also accompanied by an increase in C=O stretching bandwidth, and that somewhat larger increases in bandwidths are consistently observed with the EChol-containing bilayers (see Table 1). However, similar patterns of sterol-induced increases in bandwidths are also observed with virtually all other phospholipid absorption bands (data not shown), indicating that this phenomenon is probably attributable to a sterol-induced increase in the mobility the phospholipid molecule as a whole. This observation is consistent with the expected sterol-induced disruption of lateral packing interactions of phospholipid molecules in the bilayer, and suggests that EChol may be more disruptive of such interactions than Chol. The possible reasons why EChol may be more effective at inducing such changes will be explored in the discussion below.

#### 4. Discussion

Although the thermotropic phase behaviour of Chol/DPPC mixtures has been studied previously by ourselves [11,46] and others [61–63] using various high sensitivity DSC instruments, we repeated these measurements on a higher sensitivity calorimeter in order to insure that a valid direct comparison between the effects of Chol and EChol on DPPC MLVs was possible. Indeed, although the present measurements of the effects of Chol on the main phase transition did not differ qualitatively or quantitatively from our previous studies, the present study does establish that the pretransition is actually not completely abolished until the Chol content exceeds 10 mol%, whereas previous studies reported that the pretransition could not be detected once Chol levels exceed 5–6 mol% (but see [52]). We ascribe this difference in findings to the higher sensitivity of the DSC instrument utilized here, as well as to an improved instrumental operating protocol, as described earlier. We note in this regard that a previous X-ray diffraction study of Chol/DPPC mixtures also found that the pretransition was abolished at 10 mol% cholesterol, when both the  $L_{\beta}'$  and  $P_{\beta}'$  phases with their tilted hydrocarbon chains, which exist in the absence of cholesterol, are completely replaced by a slightly disordered  $L_{\beta}$ -like gel phase with untilted hydrocarbon chains [64].

The effects of Chol and EChol on the thermotropic phase behaviour of DPPC bilayers differ quantitatively, but not qualitatively, at all sterol concentrations, despite their rather similar chemical structures. In particular, EChol reduces both the temperature, enthalpy and cooperativity of the pretransition of DPPC with increasing sterol concentration more markedly than does Chol. As a result, the DPPC pretransition is abolished entirely at an EChol concentration of only ~7 mol% sterol, whereas the pretransition persists until 10 mol% sterol in Chol/DPPC mixtures. The generally similar qualitative effects of both sterols on the DPPC pretransition is not unexpected, since the incorporation of low levels of both sterols would abolish the hydrocarbon chain tilt required to provide a match between the smaller cross-sectional areas of the hydrocarbon chains relative to the larger cross-sectional area of the DPPC polar headgroup in the gel state, as both sterol molecules have a very small polar headgroup. However, the intrinsically larger cross-sectional area of the EChol molecule [27,28] will make it a more effective spacer of DPPC molecules in the gel-state bilayer, thus relieving the mismatch in the cross-sectional areas of the hydrocarbon chains and DPPC polar headgroups at a lower sterol concentration than does Chol. Moreover, if the EChol molecule is indeed tilted away from the bilayer normal in the gel state, whereas the Chol molecule is not [30,31], the EChol molecule will create more disorder in the adjacent phospholipid hydrocarbon chains, thus

further augmenting the expansion of the DPPC gel-state bilayer and the accommodation of the relatively large polar headgroups of these phospholipid molecules. However, our FTIR results suggest that EChol in fact does not disorder the hydrocarbon chains of the DPPC molecule in the gel state as compared to Chol.

EChol also has a similar augmented effect on the sharp component of the DSC endotherm, reducing the temperature, enthalpy and cooperativity of this component more rapidly than does Chol at lower sterol concentrations, but less rapidly than Chol at higher sterol concentrations. Thus, the sharp component of the DSC endotherm is abolished at an EChol concentration in the DPPC bilayer above 30 mol% sterol, whereas the sharp component of the DSC endotherms persists only to about 22 mol% in Chol/DPPC binary mixtures. Since the sharp component of the DSC endotherm probably arises from the fairly cooperative hydrocarbon chain melting of domains highly enriched in DPPC and depleted of sterol [11,46], the results obtained at lower sterol concentrations could be explained by a greater perturbation by EChol relative to Chol of the gel-state organization of these DPPC-enriched domains, by a decrease in the size of these domains, or by a combination of both factors. Again, these effects could be explained by the greater size and possibly by the tilt of the EChol molecule in a similar manner to that proposed above for the differential effects of EChol and Chol on the pretransition. However, such factors would not explain the attenuated effect of EChol relative to Chol at higher sterol concentrations. Instead, the difference in the effects of EChol and Chol on the sharp component of the DPPC main transition at higher sterol concentrations could be rationalized if EChol exhibits a lower solubility than Chol in DPPC bilayers at higher concentrations, as suggested by some previous work [28,38,65].

The effects of the incorporation of EChol and Chol on the broad component of the DSC heating runs, which probably arise from the less cooperative chain melting of domains of DPPC enriched in sterol, also differ quantitatively, particularly at higher sterol concentrations. In the Chol/DPPC mixtures, the temperature of the broad component monotonically increases with increasing sterol concentration whereas the enthalpy and cooperativity decrease, until no cooperative phase transition can be detected at 50 mol% sterol. Although a similar qualitative effect is noted with EChol at lower sterol concentrations, these effects are attenuated at higher sterol concentrations relative to Chol. Moreover, the cooperative phase transition in DPPC bilayer is not totally abolished by the addition of 50 mol% EChol. These results also argue that for a limited miscibility of EChol with DPPC bilayers at higher sterol concentrations, as otherwise the intrinsically, and also possibly effectively larger EChol molecule would be expected to abolish both the sharp and broad components of the DPPC chain-melting phase transition at lower rather than higher concentrations of sterol in comparison to Chol itself. Moreover, as the differential effects of EChol and Chol on the thermotropic phase behaviour of DPPC begin to manifest themselves at around 30 mol% sterol, this observation suggests that the miscibility limit of EChol may lie in this general concentration regime, as is also supported by the unpublished X-ray diffraction information in [38], which indicate a maximum of EChol solubility in OPPC bilayers near 25 mol%. Moreover, the more limited incorporation of EChol relative to Chol in synthetic phospholipid [28,38] and *A. laidlawii* lipid dispersions [65] reported previously is also compatible with this hypothesis.

We have recently published a comparative DSC and FTIR spectroscopic study of the effects of Chol and lanosterol on the thermotropic phase behaviour and organization of DPPC bilayers [52]. Lano is the biosynthetic precursor of Chol and differs in having three, rather than two axial methyl groups projecting from the rough  $\beta$ -face of the planar steroid ring system and one axial methyl group projecting from the smooth  $\alpha$ -face, whereas Chol has none. However, both Chol and Lano are C3- $\beta$ -sterols having an axially located hydroxyl group. Our DSC studies indicate that the incorporation of Lano is more effective than Chol in reducing the enthalpy of the pretransition. Lano is also

initially more effective than Chol in reducing the enthalpies of both the sharp and broad components of main phase transition. However, at sterol concentrations of 50 mol%, Lano, unlike Chol, does not abolish the cooperative hydrocarbon chain-melting phase transition. Moreover, at higher Lano concentrations (~30–50 mol%), both sharp and broad low-temperature endotherms appear in the DSC heating scans, suggestive of the formation of Lano crystallites, and of the lateral phase separation of Lano-enriched phospholipid domains, respectively, at low temperatures, whereas such behaviour is not observed with Chol at comparable concentrations. Our FTIR spectroscopic studies demonstrate that Lano incorporation produces a less tightly packed bilayer than does Chol which is characterized by increased hydration in the glycerol backbone region of the DPPC bilayer. These and other results indicate that Lano is less miscible in DPPC bilayers than is Chol, but perturbs their organization to a greater extent, probably due primarily to the rougher faces and larger cross-sectional area of the Lano molecule and perhaps secondarily to its decreased ability to form hydrogen bonds with adjacent DPPC molecules. Nevertheless, Lano does appear to produce a lamellar liquid-ordered phase in DPPC bilayers, although this phase is not as tightly packed as in the case of Chol.

It is instructive to compare the effects of EChol and Lano on the thermotropic phase behaviour of DPPC bilayers. Both sterols behave similarly in that they are more effective at abolishing the pretransition and the main phase transition of DPPC than Chol. Moreover, both sterols increase the hydration or degree of H-bonding in the glycerol backbone region of the DPPC molecule. However, EChol and Lano also exhibit qualitative differences in their effects of DPPC bilayers. For example, EChol induced only two phases in DPPC dispersions, a sterol-poor and a sterol-rich phase, whereas Lano can induce two different sterol-rich phases at high sterol concentrations. Moreover, EChol does not appear to form a separate sterol crystallite phase at higher sterol concentrations in DPPC bilayers, whereas Lano does form such a phase. Also, EChol does not seem to exhibit significant immiscibility with DPPC until sterol concentrations exceed about 30 mol%, whereas Lano exhibits immiscibility at lower sterol concentrations. Finally, at 50 mol% sterol addition, EChol reduces the total enthalpy of the main transition of DPPC by about 90%, where as Lano is less effective in this regard. These comparative results indicate that the three additional methyl groups present on the Lano steroid nucleus have a considerably greater effect on disrupting the thermotropic phase behaviour of DPPC dispersions, and of reducing sterol miscibility of DPPC bilayers, than does inverting the stereochemical orientation of the C3-hydroxyl group, although the epimerization of the C3-hydroxyl of Chol clearly also produces significant effects of sterol/DPPC interactions. Moreover, the fact that EChol does not appear to form a separate sterol crystalline phase at high sterol concentrations and continues to reduce the overall enthalpy of the DPPC main phase transition above 30 mol%, suggests that EChol may form dimers, rather than large aggregates, at high sterol concentrations. Such dimers could be stabilized by H-bonding between the C3- $\alpha$ -OH groups of the EChol molecule projecting out from the plane of the planar steroid nucleus, since the hydroxyl groups of “back-to-back” EChol molecules would be well localized and properly oriented for such interactions. In contrast, the dimerization of Chol molecules would be reduced by the orientation of the C3- $\beta$ -hydroxyl groups in the plane of the steroid nucleus and by their localization nearer the DPPC polar headgroups, where H-bonding to the DPPC phosphate and carbonyl groups would be favored [66,67]. In this regard, it is significant that the magnitude of the condensing effect of EChol in DPPC monolayers is attenuated above 30 mol%, but still continues to increase as EChol levels reach 50 mol%, again suggesting the lack of a sharp miscibility limit in this system [28].

We can compare the present DSC and FTIR spectroscopic results for EChol/DPPC mixtures with those in the previous literature in order to understand the possible basis for the rather disparate findings often

reported in comparative studies of the effects of Chol and EChol on various phospholipid model membrane systems. Our DSC results agree qualitatively, but not quantitatively, with those of de Kruffy et al. [38], who reported that EChol is less effective than Chol in reducing the enthalpy and cooperativity of main phase transition of phospholipid bilayers and that EChol is essentially immiscible above about 25 mol% sterol, such that a maximum reduction in the enthalpy of the gel/liquid-crystalline phase transition of only 50% is possible. In contrast, we find that although EChol does interact less strongly with phospholipid bilayers at sterol concentrations above 30 mol%, EChol perturbs the main phase transition only moderately less strongly than Chol and that 50 mol% EChol reduces the enthalpy of the main phase transition by about 90%. In this regard, our calorimetric results agree closely with those of George and McElhaney [68] on *A. laidlawii* ( $\text{Na}^+ + \text{Mg}^{2+}$ )-ATPase-containing DMPC LUVs. This difference in results is almost certainly due primarily to the use of a low-sensitivity DSC instrument in the former study and high-sensitivity calorimeters in this and the latter study, as well as our use of an experimental protocol designed to detect and accurately quantitate the less energetic and less cooperative chain-melting phase transitions occurring at high sterol concentrations. Moreover, the differences between these two studies may also be explained by the fact that de Kruffy et al. [38] utilized the unsaturated phospholipid OPPC, whereas the other two studies utilized the saturated phospholipids DMPC or DPPC. Since Chol is known to be less soluble in highly unsaturated phospholipid bilayers (see [50,69]) and that such effects may be exaggerated in the case of sterol analogs [47,52], the difference in the degree of saturation of the phospholipid system utilized could also have contributed to the difference in results between the older and the two newer studies.

In view of the results of the present DSC and FTIR results, which generally indicate that EChol behaves rather similarly to Chol but whose effects on the host DPPC bilayer are moderately attenuated, we can re-examine the literature in order to resolve and rationalize some of the apparent discrepant results discussed in Introduction. In this regard, it is likely that the later monolayer results of Stottrup and Keller [28] and Stine et al. [29], who found that EChol is nearly as effective overall as Chol in condensing DPPC or DMPC monolayers, respectively, are more likely to be correct than the results of the earlier study of Demel et al., who found that EChol is much less effective than Chol in condensing equimolar DPPC/sterol monolayers. Similarly, the NMR studies reviewed in the Introduction [30–33], which generally found that EChol was only slightly less effective than Chol in ordering the hydrocarbon chains of DMPC, DPPC or egg PC bilayers in the liquid-crystalline state and somewhat more effective than Chol in disordering the hydrocarbon chains in the gel state, are more likely to be correct than the ESR studies [33,34], which found that EChol incorporation was essentially without effect in bilayers composed of natural membrane lipids or egg PC. As well, the observation of only a slightly attenuated ability of EChol, as compared to Chol, to induce  $L_0$  domain formation in binary or ternary lipid monolayer and bilayer model membranes [28,41,42] seems compatible with the results of our present study and with most of those in the literature. Similarly, the greater effect of EChol as compared to Chol in reducing the  $L_{\alpha}/H_{II}$  phase transition temperature of unsaturated PEs seems reasonable, in that the greater cross-sectional area of the former sterol, and its attenuated effect on ordering phospholipid hydrocarbon chains, should result in EChol preferentially stabilizing the curved inverted relative to the more planar lamellar phase [43]. Finally, the molecular dynamics study of Rog and Pasenkiewicz-Gierula [44], which found that EChol exhibits similar and only moderately weaker effects on DMPC bilayers compared to Chol, is also compatible with our results, as well as with most of the monolayer and all of the NMR results just summarized.

A similar evaluation of the rather discrepant results of the comparative effects of EChol and Chol on the functional properties of various phospholipid bilayers can also be attempted. In this regard,

the findings of Demel et al. [35], that EChol is almost as effective as Chol in reducing  $\text{Rb}^+$  release from sonicated egg PC vesicles, and those of Bittman and Blau [36], who found that EChol had only a slightly smaller effect than Chol on reducing the water permeability of similar vesicles, seem reasonable. On the other hand, the report by the former workers [35], that EChol incorporation had little effect on the rate of glucose leakage or glycerol entry into sonicated egg PC vesicles, whereas Chol incorporation reduced glucose and glycerol permeation drastically, seem difficult to reconcile, as is the later report by Clejan et al. [37], who found that EChol incorporation actually slightly increased the rate of glucose release from sonicated dialkyl PC vesicles. Finally, the report of de Kruffy et al. [38], that the incorporation of EChol into *A. laidlawii* membranes has no effect on glycerol and erythritol permeation, whereas the incorporation of comparable amounts of Chol reduces the rate of permeation by about half, is also difficult to rationalize. Similarly, the findings of George and McElhaney [68], that the incorporation of Chol and EChol into DMPC LUVs have qualitatively similar but quantitatively different effects of the activity of the *A. laidlawii* ( $\text{Na}^+ + \text{Mg}^{2+}$ )-ATPase, but with Chol being somewhat more effective at reducing ATPase activity in the liquid-crystalline state and somewhat less effective at increasing activity in the gel state, are fully compatible with the present and with most of the previous results. However, the report of de Kruffy et al. [65], that the incorporation of EChol into the *A. laidlawii* membrane has no effect on the activity or temperature dependence of the ( $\text{Na}^+ + \text{Mg}^{2+}$ )-ATPase, is difficult to reconcile. The reason for the latter results not agreeing with the former may be because the latter study did not employ assay conditions that insured that the true  $V_{\text{max}}$  of this ATPase was being measured [70,71].

The results of the studies of Nakagawa et al. [39] and Kan et al. [40], who both report a much faster rate of spontaneous exchange between sonicated phospholipid vesicles for EChol as compared to Chol, also seem difficult to reconcile with those of this and most previous studies. These groups interpret their results as indicating that EChol interacts much more weakly with adjacent phospholipid molecules in the host phospholipid bilayer than does Chol. However, the molecular dynamics simulations of Rog and Pasenkiewicz-Gierula [44] indicate that the number of hydrogen bonds and charged-pair interactions between the incorporated sterol and adjacent DMPC molecules differ only modestly between EChol and Chol, as do the relative strengths of the van der Waals interactions between the steroid nucleus and the DMPC hydrocarbon chains. This finding would seem incompatible with more than a 10-fold difference in their exchange rates. Although the shallower localization of EChol in phospholipid bilayers suggested by this molecular dynamics study may also contribute to the much higher exchange rate of EChol compared to Chol, the large magnitude of the difference in exchange rate still seems difficult to fully rationalize. Since the sterol contents of the sonicated egg PC and DPPC vesicles used for the sterol exchange studies were not overly high (33 and 24 mol%, respectively), it would not seem that EChol would be above its solubility limit, particularly in the latter system, so a differential miscibility of Chol and EChol in these vesicles systems would not appear to explain the large difference in their spontaneous exchange rates, unless the EChol solubility is differentially reduced relative to Chol in small unilamellar vesicles as compared to MLVs or LUVs of large diameter.

In summary, our present results, and most of those in the previous literature, support the view that C3- $\beta$ -hydroxy sterols such as Chol are somewhat more effective than C3- $\alpha$ -hydroxy sterols such as EChol in their ability to exert their characteristic Chol-like effects on the host phospholipid bilayer. However, neither our present results, nor most of the findings of others, support the view that EChol has strongly attenuated or even no effects on the host phospholipid bilayer. In addition, although our results do support the idea that the effects of EChol on the host phospholipid bilayer are further attenuated at higher sterol concentrations, our findings do not indicate a sharp

miscibility limit and the formation of a separate sterol phase, at least in DPPC bilayers. However, it seems clear that additional experimental work will be required to address currently open questions, as well as some of the apparently discrepant results in the current literature.

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